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# **Deletion of calcineurin from GFAP-expressing astrocytes impairs excitability of cerebellar and hippocampal neurons through astroglial Na<sup>+</sup>/K<sup>+</sup> ATPase.**

Running title: Neuronal hypoexcitability in astroglial CaN KO

Laura Tapella<sup>1#</sup>, Teresa Soda<sup>2#</sup>, Lisa Mapelli<sup>2</sup>, Valeria Bortolotto<sup>1</sup>, Heather Bondi<sup>1</sup>, Federico A Ruffinatti<sup>1</sup>, Giulia De Matteis<sup>1</sup>, Alessio Stevano<sup>1</sup>, Marianna Dionisi<sup>1</sup>, Simone Ummarino<sup>3,4</sup>, Annalisa Di Ruscio<sup>3,4</sup>, Carla Distasi<sup>1</sup>, Mariagrazia Grilli<sup>1</sup>, Armando A Genazzani<sup>1</sup>, Egidio D'Angelo<sup>2\*</sup>, Francesco Moccia<sup>5</sup>, Dmitry Lim<sup>1\*</sup>

## **Affiliations**

<sup>1</sup> Department of Pharmaceutical Sciences, Università del Piemonte Orientale “Amedeo Avogadro”, Via Bovio 6, 28100, Novara, Italy;

<sup>2</sup> Department of Brain and Behavioral Sciences, University of Pavia, Via Forlanini 6, 27100, Pavia, Italy;

<sup>3</sup> Center of Life Science, Medical School Initiative for RNA Medicine, Harvard Medical School, 3 Blackfan Circle, Boston, 02115, MA, USA;

<sup>4</sup> Department of Translational Medicine, Università del Piemonte Orientale, via Paolo Solaroli 17, 28100, Novara, Italy;

<sup>5</sup> Department of Biology and Biotechnology “Lazzaro Spallanzani”, University of Pavia, Via Forlanini 6, 27100, Pavia, Italy;

\* Correspondence should be sent to: Egidio D'Angelo, [egidiougo.dangelo@unipv.it](mailto:egidiougo.dangelo@unipv.it); Dmitry Lim, [dmitry.lim@uniupo.it](mailto:dmitry.lim@uniupo.it), Tel.: +39-0321 375822

# These Authors contributed equally.

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The authors have no competing interests.

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## ABSTRACT

Astrocytes perform important housekeeping functions in the nervous system including maintenance of adequate neuronal excitability, although the regulatory mechanisms are currently poorly understood. The astrocytic  $\text{Ca}^{2+}$ /calmodulin-activated phosphatase calcineurin (CaN) is implicated in the development of reactive gliosis and neuroinflammation, but its roles, including the control of neuronal excitability, in healthy brain is unknown. We have generated a mouse line with conditional knockout (KO) of CaN B1 (CaNB1) in glial fibrillary acidic protein (GFAP)-expressing astrocytes (astroglial calcineurin knock-out, ACN-KO). Here we report that postnatal and astrocyte-specific ablation of CaNB1 did not alter normal growth and development as well as adult neurogenesis. Yet, we found that specific deletion of astrocytic CaN selectively impairs intrinsic neuronal excitability in cerebellar granule cells (CGCs) and hippocampal CA1 pyramidal neurons. This impairment is associated with a decrease in after-hyperpolarization in CGC, while passive properties of both CGCs and CA1 pyramidal neurons were unchanged, suggesting impairment of  $\text{K}^+$  homeostasis. Indeed,  $\text{Na}^+/\text{K}^+$  ATPase (NKA) activity was significantly lower in cerebellar and hippocampal lysates and in pure astrocytic cultures from ACN-KO mice. While no changes were found in mRNA or protein levels of NKA brain isoforms, NKA activity was also inhibited by the specific CaN inhibitor FK506 in both cerebellar lysates and primary astroglia from WT mice, suggesting that calcineurin directly modulates NKA activity and in this manner controls neuronal excitability. In summary, our data provides formal evidence for the notion that astroglia is fundamental for controlling basic neuronal functions and place calcineurin center-stage as a astrocytic  $\text{Ca}^{2+}$ -sensitive switch.

## 1. INTRODUCTION

The  $\text{Ca}^{2+}$ /calmodulin (CaM)-activated serine-threonine phosphatase, calcineurin (CaN), is abundantly expressed in neurons (Klee et al., 1979) but also in other brain cellular types, including astrocytes (Lim et al., 2016). CaN is a heterodimer composed of a larger (59 kDa) catalytic subunit A (CaNA) and a smaller (19 kDa) obligatory regulatory subunit B (CaNB). Three genes (Ppp3ca, Ppp3cb and Ppp3cc) encode for CaNA ( $\alpha$ ,  $\beta$  and  $\gamma$ ) and two genes (Ppp3r1 and Ppp3r2) encode for CaNB (1 and 2). Two catalytic (CaNA $\alpha$  and CaNA $\beta$ ) and one regulatory (CaNB1) subunits are expressed in the brain and therefore the selective suppression of CaN activity in mouse models is routinely achieved by deletion of the regulatory CaNB1 subunit (Zeng et al., 2001; Miyakawa et al., 2003).

CaN is activated by an increase in cytosolic  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ) and it has been shown that low but long-lasting elevations of  $[\text{Ca}^{2+}]_{\text{cyt}}$  specifically activate CaN (Negulescu et al., 1994; Dolmetsch et al., 1997). Neuronal CaN plays a crucial role in the control of neurotransmission, synaptic plasticity and memory formation (Zeng et al., 2001; Baumgärtel et al., 2008). Accordingly, it has long been known that CaN finely regulates the strength of synaptic transmission by eliciting long-term depression (LTD) and by constraining long-term potentiation (LTP) or resetting potentiated synapses (Baumgärtel and Mansuy, 2012). In addition, endogenous CaN may modulate neuronal excitability. For instance, an increase in CaN activity reduces the firing rate by up-regulating the A-type  $\text{K}^+$  channel, Kv4.2 in mouse pyramidal neurons (Yao et al., 2016) and by shortening the axon initial segment in mouse dentate granule cells (Evans et al., 2015). Although expressed at significantly lower levels in astrocytes compared to neurons, astrocytic CaN is upregulated and plays a crucial role in astrogliosis and neuroinflammation (Pekny et al., 2016). For instance, astroglial CaN regulates neuronal excitability in a murine model of Alzheimer's Disease (Sompol et al., 2017) and modulates synaptic strength and long-term plasticity in a rat model of traumatic brain injury (Furman et al., 2016). However, there is no evidence about the role of astroglial CaN in neuronal excitability in the healthy brain.

Here, we report the generation and characterization of a mouse model with conditional CaNB1 KO specific for glial fibrillary acidic protein (GFAP)-expressing astrocytes (astroglial calcineurin knock-out, ACN-KO). We demonstrate that ACN-KO mice do not present alterations of development, growth, brain weight and architecture and therefore are suitable for studying the role of astrocytic CaN in healthy brain. We further report that ACN-KO mice display an impairment of excitability of cerebellar granule cells (CGCs) and CA1 hippocampal pyramidal neurons and provide evidence that this may be due to the functional inactivation of the astroglial  $\text{Na}^+/\text{K}^+$  ATPase. These findings provide the first evidence that astrocytic CaN modulates neuronal excitability in the healthy brain, a novel mechanism which bears remarkable consequences on information processing at the tripartite synapse.

## 2. MATERIALS AND METHODS

### 2.1. Generation and handling of ACN-KO mice

Conditional CaN knockout (KO) in astroglial cells was generated by crossing CaNB1<sup>flox/flox</sup> mice (Neilson et al., 2004) (Jackson Laboratory strain B6;129S-Ppp3r1tm2Grc/J, stock number 017692), in which 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> exons in the CaNB1, a regulatory CaN subunit gene, were flanked by LoxP sequences, with mice in which expression of bacterial Cre recombinase was controlled by the full-length murine GFAP promoter (Gregorian et al., 2009) (Jackson Laboratory strain B6.Cg-Tg(Gfap-cre)77.6Mvs/2J, stock number 024098). F1 founder mice were backcrossed for four generations and the line was maintained by crossing CaNB1<sup>flox/flox</sup>/GfapCre<sup>-/-</sup> (thereafter referred as to ACN-Ctr) males with CaNB1<sup>flox/flox</sup>/GfapCre<sup>+/-</sup> (ACN-KO) females (Supplementary Fig. 1A).

To generate ACN-KO mice expressing tdTomato reporter, ACN-KO mice were crossed with Rosa26-CAG-flox-stop-flox-tdTomato (RATO) mice (kind donation from Prof. Frank Kirchhoff, CIPMM, Center for Integrative Physiology and Molecular Medicine, Molecular Physiology, University of Saarland, Homburg) resulting RATO<sup>flox/flox</sup>/CaNB1<sup>flox/flox</sup>/GfapCre<sup>+/-</sup> mice (thereafter referred as to RATO-ACN-KO) and RATO<sup>flox/flox</sup>/CaNB1<sup>wt/wt</sup>/GfapCre<sup>+/-</sup> (RATO-ACN-Ctr) (Supplementary Fig. 1B). Genotyping was performed by means of PCR and real-time PCR. Genotyping protocols are provided in Supplementary Methods section.

Mice were housed in the animal facility of the Università del Piemonte Orientale, were kept at three to four per cage, and had unlimited access to water and food. Animals were managed in accordance with European directive 2010/63/UE and with Italian law D.l. 26/2014. The procedures were approved by the local animal-health and ethical committee (Università del Piemonte Orientale) and were authorized by the national authority (Istituto Superiore di Sanità; authorization numbers N. 77-2017 and N. 214-2019). All efforts were made to reduce the number of animals by following the 3R's rule.

### 2.2. Histochemistry

Ctr and ACN-KO mice were perfused transcardially, brains were collected, and processed as previously described (Denis-Donini et al., 2008). Sagittal 40- $\mu$ m-thick cryosections have been prepared and used for immunostaining. For DCX immunolabelling (n = 4/group), 3 sections/mouse have been selected, spaced 640  $\mu$ m in order to analyze different levels along the entire extension of dentate gyrus formation.

#### 2.2.1. Neurogenesis assessment

To analyze the neuronal differentiation of new-born cells, ACN-Ctr and ACN-KO mice (n=5/group) were intraperitoneally injected with bromodeoxyuridine (BrdU) (150 mg/kg of body weight i.p.; Sigma-Aldrich, Saint Louis, MO) for three consecutive days. Mice were sacrificed thirty days after the last BrdU injection. For immunofluorescence with antibodies against BrdU and NeuN a series of one-in-eight sections through the dentate gyrus was selected and analyzed as previously described (Denis-Donini et al., 2008). Primary antibodies were as follow: goat anti- doublecortin (DCX) antibody (1:300; Santa Cruz Biotechnology, Santa

Cruz, CA), rat anti-BrdU antibody (1:200; Novus Biologicals Inc., Littleton, CO), mouse anti-neuronal nuclei (NeuN) antibody (1:150; Millipore Corp., Billerica, MA).

SOX2 and NeuN stainings

### **2.2.1. Anti-CaNB1 staining**

For anti-CaNB1 immunohistochemistry cryosections were first subjected to antigen retrieval and probed with rabbit anti-CaNB1 antibody (1:200; Millipore Corp. Cat. 07-1494). Chicken anti-rabbit Alexa Fluor 488 secondary antibody was used. Confocal images were taken using Zeiss 710 confocal laser scanning microscope equipped with N-Achroplan 10x/0.25 or EC Plan-Neofluar 40x/1.30 Oil DIC M27 objectives and Zen software. Standard deviation of the intensity profile of anti-CaNB1 stained sections was calculated using MetaMorph software (Molecular Devices, Sunnyvale, CA, US).

### **2.3. FACS isolation of astrocytes**

For isolation of astrocytes by FACS, P20 ACN-KO and ACN-Ctr pups were anesthetized and sacrificed by decapitation. Cerebella were dissected in cold HBSS (Sigma, Cat. H6648), minced and digested in 0.125% Trypsin (Sigma, Cat. T3924) in presence of 20 u of DNase (Sigma, Cat. D5025) at 37°C with gentle agitation for 30 min. Cerebella from 2 animals were processed in a single 2 ml Eppendorf tube. After brief centrifugation (3 min 300 xg) tissues were resuspended in 1.5 ml HBSS supplemented with 3% fetal bovine serum (FBS, Gibco, Cat. 10270) and 5 mM EDTA (HBSS-FBS-EDTA), and gently triturated with 15-20 strokes of fire polished Pasteur pipettes of three decreasing sizes. After each round of trituration the suspension was left for 2 min and 1 ml was transferred into a new 15 ml Falcon tube and fresh HBSS-FBS-EDTA was added. After three rounds of trituration, the suspension was filtered through 40 µm nylon strainer (Corning, Cat. 352340). The cells were centrifuged twice at 133 xg for 8 min and resuspended in 500 µL HBSS-FBS-EDTA. For immunostaining, 100 µL aliquots were stained with Draq5 (2 µM, Thermo Fisher, Milan, Italy, Cat. 62251), anti GLAST(ACSA-1) PE-conjugated antibody (1:250, Cat 130-118-483) and with anti-CD90.2 FITC-conjugated antibody (Cat. 130-102-452) (last two from Miltenyi Biotech, Bologna, Italy). Staining was performed at 4°C for 20 min in dark. After staining cells were washed by 1 ml of HBSS-FBS-EDTA, passed through 40 µm strainer and analyzed on S3e Cell Sorter (Bio-Rad, Segrate, Italy). Gating parameters were: singlets → Draq5<sup>+</sup> (FL4) → GLAST-PE<sup>+</sup> or tdTomato<sup>+</sup> (FL2) → CD90.2<sup>-</sup> (FL1) (Suppl. Figure 3). From 4x10<sup>4</sup> to 2x10<sup>5</sup> cells were routinely sorted from one preparation directly in Trizol-LS (Invitrogen, Cat. 10296028).

### **2.4. Primary astroglial and mixed cultures**

Primary cultures were prepared as described previously (Tapella et al., 2018) with slight modifications. In brief, for purified astroglial cultures, cerebella were dissected from 3- to 5-day-old pups in cold HBSS, digested in 0.25% trypsin (37 °C, 20 min), washed in complete culture medium (Dulbecco's modified Eagle's medium (DMEM, Cat. D5671), supplemented with 10% fetal bovine serum (FBS), and with 2 mg/mL glutamine, 10 U/mL penicillin and 100 lg/mL streptomycin (all from Sigma)) and resuspended in cold HBSS supplemented

with 10% FBS. After 30 strokes of dissociation with an automatic pipette, cell suspension was centrifuged (250 x g, 5 min), pellet was resuspended in 4 ml complete medium and seeded in 65-mm Falcon culture dishes, pretreated with 0.1 mg/mL Poly-L-lysine (Cat. P2636, Sigma). Each pup was processed separately. At sub-confluence (5–10 days in vitro; DIV), cells were detached with trypsin and plated for experiments. At this stage, microglial cells were removed by magnetic-activated cell sorting (MACS) negative selection using anti-CD11b-conjugated beads (Cat. 130-093-636) and MS magnetic columns (Cat. 130-042-201), (both Miltenyi Biotec, Bologna, Italy). As controlled by anti-GFAP (astroglial marker), anti-Iba1 (microglial marker) and anti-MAP2 (neuronal marker, all from Merck Millipore, Billerica, MA) immunocytochemistry, resulting cultures contained virtually no microglial and neuronal cells (Ruffinatti et al., 2018).

Mixed astrocyte-neuron cultures were prepared as described previously (Grolla et al., 2013) with slight modifications. After enzymatic and mechanical dissociation, final cellular pellet was resuspended in neurobasal A medium (Invitrogen, Cat. 10888022) supplemented with 2% B27 supplement (Invitrogen, Cat. 17504044), 2 mg/mL glutamine, 10 U/mL penicillin and 100 mg/mL streptomycin, and plated as described above. Two-third of medium volume was changed every 5<sup>th</sup> day and the cells were lysed at days in vitro (DIV) 12-15. For enriched neuronal cultures, cytosine  $\beta$ -D-arabinofuranoside (40  $\mu$ M, Cat. C6645, Sigma) at DIV4 and neurons were lysed at DIV12.

## **2.5. Total RNA extraction and real-time PCR**

Tissues or cells were lysed in Trizol reagent (Invitrogen, Cat. 15596026). Total RNA was extracted using Absolutely RNA miRNA kit (Agilent, 400809) according to manufacturer's instructions. 0.5-1  $\mu$ g of total RNA was retrotranscribed using random hexamers and ImProm-II RT system (Promega, Milan, Italy, Cat. A3800). Real-time PCR was performed using iTaq qPCR master mix according to manufacturer's instructions (Bio-Rad, Segrate, Italy, Cat. 1725124) on a SFX96 Real-time system (Bio-Rad). To normalize raw real-time PCR data, S18 ribosomal subunit was used. Sequences of oligonucleotide primers is listed in Supplementary Table 1. The real-time PCR data are expressed as delta-C(t) of gene of interest to S18 allowing appreciation of the expression level of a single gene.

## **2.6. Lysates preparation and Western blotting**

Astrocytes were grown in 100 mm dishes (1x10<sup>6</sup> /dish), while mixed neuron-astroglial cultures were grown in 65 mm dishes- (0.5 mln cells/dish). Cells were lysed (lysis buffer: 50 mM Tris HCl (pH 7.4), sodium dodecyl sulfate (SDS) 0.5%, 5 mM EDTA) and 20  $\mu$ g proteins were resolved on 12% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane using Trans-Blot Turbo Transfer System (Bio-Rad), blocked in skim milk (5%, for 1 h; Cat. 70166; Sigma) and immunoblotted with indicated antibody. Rabbit anti-GAPDH (1:5000, Sigma Cat. G9545) or anti- $\beta$ -actin (1:10000, Sigma, Cat. A1978) were used to normalize protein load. Primary antibodies were: rabbit anti-CaNB1 (1:500; Millipore Corp. Cat. 07-1494); mouse anti-PSD95, clone K28/43, antibody (1 : 1000, Cat. MABN68, Merck Millipore, Vimodrone, Italy); mouse anti-Sy38 (1:500, Immunological Sciences, Cat. MAB-10321). Goat anti-mouse IgG (H+L) horseradish

peroxidase-conjugated secondary antibody (1 : 8000; Cat. 170-6516, Bio-Rad) was used. The protein bands were developed using SuperSignal West Pico chemiluminescent substrate (Cat. 34078; Thermo Scientific, Rockford, IL, USA). Densitometry analysis was performed using Quantity One software (Bio-Rad).

## **2.7. Electrophysiology**

### ***2.7.1. Cerebellum Slice preparation and cerebellar granule cells electrophysiology***

The mice were anesthetized with halothane (Sigma, St. Louis, MO) and killed by decapitation in order to remove the cerebellum for acute slice preparation according to a well-established technique (Armano et al., 2000; Sola et al., 2004). The cerebellar vermis was isolated and fixed on the vibroslicer's stage (Leica VT1200S, etc) with cyano-acrylic glue. Acute 220  $\mu\text{m}$ -thick slices were cut in the parasagittal plane in cold cutting solution containing (in mM): 130 potassium gluconate, 15 KCl, 0.2 EGTA, 20 Hepes and 10 glucose, pH adjusted to 7.4 with NaOH. Slices were incubated for at least 1 h at room temperature (24–25°C) in oxygenated extracellular Krebs solution containing (in mM): 120 NaCl, 2 KCl, 2  $\text{CaCl}_2$ , 1.2  $\text{MgSO}_4$ , 1.2  $\text{KH}_2\text{PO}_4$ , 26  $\text{NaHCO}_3$ , 11 glucose, pH 7.4, when equilibrated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Slices were later transferred to a recording chamber mounted on the stage of an upright microscope (Zeiss, Oberkochen, Germany) and perfused (1–1.5  $\text{ml min}^{-1}$ ) with the same extracellular solution added with 10 mM Gabazine (SR95531) to block GABA-A receptors (Abcam) and maintained at 32°C. Patch pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) and, when filled with the intracellular solution, had a resistance of 7–9  $\text{M}\Omega$  before seal formation. The whole-cell recording pipettes were filled with the following solution (in mM): 126 potassium gluconate, 4 NaCl, 5 Hepes, 15 glucose, 1  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 BAPTA-free, 0.05 BAPTA- $\text{Ca}^{2+}$ , 3  $\text{Mg}_2^{+}$ -ATP, 0.1  $\text{Na}^{+}$ -GTP, pH 7.2 adjusted with KOH. This calcium buffer is estimated to maintain free calcium concentration around 100nM. We performed whole-cell patch-clamp recordings from CGCs in acute cerebellar slices. Recordings were performed with a Multiclamp 700-B amplifier (3dB; cutoff frequency (fc), 10 kHz), data were sampled with a Digidata 1440A interface, and analyzed off-line with pClamp10 software (Axon Instruments, Molecular Devices). Membrane current and potential were recorded using the voltage-clamp mode and the current-clamp mode of the amplifier, respectively (D'Angelo et al., 1995). To examine the intrinsic excitability of CGCs we used the current clamp mode of the whole-cell patch-clamp technique: CGC resting membrane potential was set at -80 mV and the cells were then injected for 2 s or 800 ms with depolarizing current steps (from 8 to 48 pA in a 2 or 4 pA increment).

### ***2.7.2. Hippocampal slice preparations and CA1 pyramidal neurons electrophysiology***

The mice were anesthetized with halothane (Sigma, St. Louis, MO) and killed by decapitation. Brains were carefully removed and placed in an ice-cold solution containing (mM): 87 NaCl, 2.5 KCl, 25  $\text{NaHCO}_3$ , 1.25  $\text{NaH}_2\text{PO}_4$ , 75 Sucrose, 25 Glucose, 0.2  $\text{CaCl}_2$ , 7  $\text{MgCl}_2$ , bubbled with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Brains were glued with cyanoacrylate glue to a support placed inside the cutting chamber of the vibratome (1000 plus, Vibratome, USA) and cut in 300  $\mu\text{m}$  coronal slices. The slices were placed in Artificial cerebrospinal fluid aCSF solution



containing (mM): 125 NaCl, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 10 Glucose, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub> for one hour at 34–35 °C and thereafter for at least one hour at room temperature before recordings (Cunha et al., 2018). We performed whole-cell patch-clamp recordings from CA1 pyramidal neurons. Recordings were performed under continuous perfusion (1–1.5 ml min<sup>-1</sup>) with a bath solution containing (in mM): NaCl (119), KCl (5), HEPES (20), glucose (30), MgCl<sub>2</sub> (2), CaCl<sub>2</sub> (2) and in the same extracellular solution added with 10 µM Gabazine (SR95531) to block GABA-A receptors (Abcam) and maintained at 32°C. To record intrinsic properties of neurons, we used patch pipettes (resistance between 5.5–7 MΩ), filled with an intracellular solution consisting of (mM): 138 K-gluconate, 8 KCl, 10 Hepes, 0.5 EGTA, 4 Mg-ATP, 0.3 Na-GTP adjusted to pH 7.3 with KOH and 300mOsm. The intrinsic excitability of CA1 was measured by using the current clamp mode of the whole-cell patch-clamp technique: CA1 resting membrane potential was set at -80 mV and the cells were then injected for 2 s or 800 ms with depolarizing current steps (50 pA in a 25 pA increment).

### **2.7.3. Basal synaptic transmission**

### **2.7.4. Resting membrane potential of cultured astrocytes**

Resting membrane potential (RMP) recordings were performed at room temperature in the whole-cell configuration using an Axopatch 200A (Axon Instruments). Cells were bathed in a solution containing (in mM): 138 NaCl, 4 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 glucose and 10 HEPES at pH 7.25 adjusted with NaOH. Whole-cell patch pipettes were filled with (in mM): 140 KCl, 2 NaCl, 5 EGTA, 0.5 CaCl<sub>2</sub> and 10 HEPES at pH 7.25 adjusted with KOH (Rocchio et al., 2019). Pipettes had a resistance of 3–5 MΩ. Soon after the patch rupture, membrane resting potential values were digitized with Digidata 1440A (Molecular Devices, USA) and recorded using pClamp software (Molecular Device, USA) at a sampling frequency of 1 kHz. A single measurement was obtained by averaging the first 30 s of the RMP trace.

### **2.8. NKA ATPase assay**

To measure NKA ATPase activity hippocampal or cerebellar tissue from one animal per point were used. Purified astroglial and enriched neuronal cultures were grown in 6 well plates at an approximate density of 1 x 10<sup>5</sup> cells per well. Cell or tissues were lysed in a ATPase/GTPase activity assay kit buffer, and free phosphate was measured as according to manufacturer's instructions (ATPase/GTPase activity assay kit, Cat. MAK113-1KT, Sigma). Ouabain (3 mM, Cat. 1076, Tocris) was added and NKA-specific ATPase activity was calculated as a difference between Ouabain and vehicle-treated samples. When whole-tissue lysates were processed, FK506 (200 nM) was added together with Ouabain. For determination of NKA ATPase activity in astroglial and neuronal cultures, FK506 was added to culture medium (100 nM) 24 h prior lysate preparation. Data are expressed as pmol of consumed inorganic phosphate per µg of total proteins per minute of reaction (pmol/µM/min).

### **2.9. NKA ATPase assay**

## 2.10. Glutamate uptake and release and lactate release measurements

## 2.11. Statistical analysis

For statistical analysis and related graphical representations, GraphPad Prism v.7 and G\*Power 3.1 (Faul et al., 2007) were used. Normally distributed data were expressed as mean  $\pm$  SEM and parametric hypothesis tests have been used whenever possible, after having checked the normality of residuals and the homoscedasticity of the dataset (through Shapiro-Wilk and Levene's test respectively). In particular, unless otherwise specified, two-sample comparisons were performed using the unpaired two-tailed Student's *t*-test, while, for comparisons between more than two experimental groups, ANOVA (one- or two-way, according to the number of independent variables) was used along with HSD Tukey post-hoc test. When the above assumptions were not met, parametric tests have been avoided in favor of specific nonparametric alternatives and median and interquartile range (IQR) were used as measures of central tendency and dispersion respectively. In any case, tests were conducted at the significance level  $\alpha=0.05$  and the exact *p*-values are reported over the scatter-bars in the graphs. Additionally, for the most critical comparisons (namely when failing in rejecting the null hypothesis using a sample size smaller than  $n=6$  per condition), a statistical power sensitivity analysis has been carried out to determine the minimum detectable effect (MDE) with  $\alpha=0.05$  and a statistical power  $\pi=0.8$ . Results of such a sensitivity analysis are reported in figure captions (Figs. 3A-B, 4E, 7B-C to be checked!) in terms of both Cohen's *d* and minimum detectable difference in dependent variable values ( $\Delta$ ), taking advantage of the estimated (pooled) standard deviation of the population(s).

Faul F., Erdfelder E., Lang A.G., Buchner A. (2007). G\*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods*, 39, 175-191.

### 3. RESULTS

#### 3.1. Generation and validation of conditional astroglial CaN KO mouse line

A mouse model with conditional KO of CaN in GFAP-expressing astrocytes was generated by crossing calcineurin B1 (CaNB1)<sup>flox/flox</sup> mice (Neilson et al., 2004) with 77.6 GFAP-Cre mice (Gregorian et al., 2009) resulting in CaNB1<sup>flox/flox</sup>:GFAP-Cre<sup>+/-</sup> (astroglial calcineurin conditional knock-out, ACN-KO) and CaNB1<sup>flox/flox</sup>:GFAP-Cre<sup>-/-</sup> (ACN-Ctr) mice (Suppl. Fig. 1). It is known that, in such brain regions as cerebellum (CB) and hippocampus (Hip), nearly all astrocytes are GFAP-positive (Verkhatsky and Nedergaard, 2018); hence, in these brain regions nearly all astrocytes will have deletion of CaN. We, therefore, used these two brain regions for validation and characterization of ACN-KO mice. At the mRNA level, primers that span the deleted region in the CaN gene (from the 2<sup>nd</sup> to the 6<sup>th</sup> exon), amplify only a 517 bp band in ACN-Ctr, while a shorter band of 95 bp appears in ACN-KO CB (Fig. 1A) and Hip (Suppl. Fig. 2A) due to elimination of exons 3-5 from the CaNB1 gene in a sub-population of cells, likely to be GFAP<sup>+</sup> astrocytes. Notably, in PCRs using primers that amplify only the deleted RNA region (exons 3-5, Fig. 1B and Suppl. Fig. 2B) amplicons were present in whole tissue preparations from both ACN-KO and ACN-Ctr with no differences in band intensity, strongly suggesting that CaNB1 expression in neurons is unaltered. As expected, furthermore, Cre mRNA was present only in ACN-KO tissues (Fig. 1C and Suppl. Fig. 2C). To formally demonstrate astrocyte-specific CaNB1 deletion, we made use of fluorescence-activated cell sorting (FACS) and isolated Draq5<sup>+</sup>/GLAST<sup>+</sup>/CD90.2<sup>-</sup> astrocytes from the CB of postnatal day 20 (P20) ACN-KO and ACN-Ctr mice (Suppl. Fig. 3). In these preparations, primers amplifying exons 3-5 failed to detect CaNB1 mRNA from FACS-purified astrocytes by real-time PCR (Fig. 1D). Abundant expression of CaN, including CaNB1, in neurons, instead, did not allow direct validation of CaNB1 KO in astrocytes by immunohistochemistry (Suppl. Fig. 4A). Nevertheless, a reduced background staining around intensely stained cerebellar Purkinje cell dendrites can be appreciated in ACN-KO as compared to ACN-Ctr tissues as revealed by measuring line scan profile and image intensity standard deviation in anti-CaNB1 stained sections (Suppl. Fig. 4B). At protein level, CaNB1 was strongly reduced in purified astroglial cultures from CB and Hip of ACN-KO mice (Fig. 1E,F) but it was not significantly changed in mixed neuron-astroglial cultures (Fig. 1G). To further demonstrate the deletion of CaNB1 in astroglial cells we crossed ACN-KO mice with Rosa26-tdTomato reporter mice (kind donation from Prof. Frank Kirchhoff, University of Saarland, Homburg, Germany, (Jahn et al., 2018)). We have enzymatically dissociated hippocampal tissues from P20 pups expressing tdTomato, plated dissociated cells, and, 48 h after plating performed immunocytochemical analysis of CaNB1 expression. We found that significantly lower expression of CaNB1 protein in ACN-KO compared with ACN-Ctr mice (Suppl. Fig. 4C).

#### 3.2. Postnatal onset of CaNB1 KO and normal neurogenesis, growth and development of ACN-KO mice

Recently, another CaNB1<sup>flox/flox</sup>/GFAP-Cre mouse line has been generated by using the human GFAP promoter. The Authors report defects in nutrition and premature death of pups around 1 mo of age (Fujita et al., 2018). Here we report that, in contrast to the mouse described by (Fujita et al., 2018), ACN-KO mice breed

normally and deliver  $6.6 \pm 2.7$  pups per litter, which is not different from that reported for the C57Bl/6 strain (Verley et al., 1967). No differences were observed in growth and body weight between ACN-Ctr and ACN-KO littermates (Fig. 2A). Likewise, there was no difference in brain weight between ACN-KO and wild type (WT) mice by 1 mo (Fig. 2B). There were no differences in the macrostructure of CB and Hip (Suppl. Fig. 5A) as well as in external appearance of ACN-KO and ACN-Ctr mice (Suppl. Fig. 5B).

To investigate the spatio-temporal progression of CaNB1 KO we followed the development of tdTomato expression by confocal microscopy on fixed ACN-KO brain samples. The expression of the reporter protein was not evident before P7, increased steeply between P7 to P15 in both CB (Fig. 2C) and Hip (Suppl. Fig. 6A) and all astrocytes in these two brain regions expressed tdTomato at P20. To verify whether there were astrocytes in which the KO has not occur, we analyzed the hippocampal formation of ACN-KO mice using the following strategy: taking into consideration that in the hippocampus astrocytes are organized in the non-overlapping three-dimensional domains and that reporter tdTomato fills in the entire volume of astrocytes, we looked for the areas with no tdTomato expression. E.g., such “empty” areas are present in the cortex, in which not all astrocytes are GFAP-positive (Suppl. Fig. 6B). As shown in Suppl. Fig. 6C, at 1 mo of age, in both ACN-Ctr and ACN-KO, the entire volume of hippocampal formations were filled with tdTomato, so that we have not able to detect clearly boundaries between astrocytic domains. This indicates that 100% of astrocytes express tdTomato and therefore, achieved the full KO.

It has been stated that 77.6 GFAP-Cre line does not have Cre expression in neuronal stem cells in the hippocampus and other brain regions except sub-ventricular zone (SVZ) (Gregorian et al., 2009). This was somewhat peculiar since a neural stem cell (NSC) marker Sox2 is typically co-expressed with GFAP during neurogenesis in peri-natal period until the second postnatal week (REFFF). It was therefore possible that there were an overlap between Sox2 and Cre expression in ACN-KO mice. In fact, we have observed that a small part of Sox2 positive cells expressed tdTomato reporter at P7 in both ACN-Ctr and ACN-KO (Suppl. Fig. 7A). If this would result Cre expression in mature neurons, we could expect numerous neuronal cells expressing tdTomato reporter later in the life. Nevertheless, at 1 mo of age in the dentate gyrus there were only a few NeuN-positive neurons expressing tdTomato (Suppl. Fig. 7B), indicating on a negligible if any effect of Cre expression in the neuronal progenitors on Cre occurrence in mature neurons.

Since adult neurogenesis impacts on neuronal excitability and functional plasticity (Lledo et al., 2006; Ikrar et al., 2013; Park et al., 2015), we quantified the number of migrating immature neurons (doublecortin (DCX) positive) and the number of newly generated neurons, (BrDU<sup>+</sup>/NeuN<sup>+</sup> cells), in the subgranular (SGZ) and in the granular cell layer (GCL) within the dentate gyrus (DG) of the hippocampal formation. No differences were found in DCX<sup>+</sup> or BrDU<sup>+</sup>/NeuN<sup>+</sup> cells between ACN-Ctr and ACN-KO preparations (Fig. 3A,B). This demonstrates that adult hippocampal neurogenesis is not impaired by the deletion of CaNB1 in GFAP-expressing astrocytes, even if subtle effects cannot be excluded because of the limited statistical sensitivity of these comparisons (see figure caption). In addition, we evaluated whether levels of pre- and postsynaptic marker proteins were different between ACN-KO and ACN-Ctr mice. As shown in Fig. 3C and D, the amounts

of synaptophysin and postsynaptic density protein 95 (PSD95) were not different in both CB and Hip tissues as well as in cultured primary hippocampal neurons.

Taken together, we have generated an astrocyte-specific KO of CaNB1 without alterations in brain structure and development that is suitable to study the role of astroglial CaN in brain physiology.

### **3.3. Impairment of intrinsic excitability in cerebellar and hippocampal neurons in ACN-KO mice**

Next, we proceeded to assess whether deletion of astroglial CaN affects neuronal intrinsic excitability in CGCs and hippocampal CA1 pyramidal neurons. Whole-cell patch-clamp recordings of CGCs (Fig. 4A) revealed that the deletion of CaN from astrocytes shifted the high-frequency tonic firing into adaptive firing while depolarizing the cells (Fig. 4B). The input-output relationship (Fig. 4C) clearly showed that, at increasing depolarizing steps, the output firing frequency (22 pA: ACN-Ctr =  $44.6 \pm 15.5$  Hz,  $n = 5$ ; ACN-KO =  $8.5 \pm 3.74$  Hz,  $n = 5$ ;  $p = 0.05$ ) was not able to follow the Ctr trend and soon dropped to a low number of spikes. In particular, ACN-KO CGCs showed rapid adaptation to high frequencies, associated to a decrease in the after-hyperpolarization (AHP) depth of the spikes during the train (Fig. 4D). While Ctr neurons were able to maintain the AHP unvaried throughout the whole discharge, KO cells showed an average decrease in the AHP of about 25% of its initial value ( $p = 0.0312$ ). Since the AHP phase of an AP is known to depend on  $K^+$  currents (D'Angelo et al., 1998), this effect might be due to a change in  $K^+$  currents or gradients in these cells. Within the statistical sensitivity determined by the limited sample size, voltage-clamp current/voltage (I/V) relationship did not show any alteration in  $Na^+$  and  $K^+$  currents (Fig. 4E). Moreover, the lower intrinsic excitability of ACN-KO granule cells was not due to the alteration of their passive properties (Table 1) including resting membrane potential. Therefore, it is reasonable to speculate that the mechanisms of  $K^+$  gradient maintenance during high frequency activation are impaired in ACN-KO CB.

The input-output relationships of hippocampal CA1 neurons (Fig. 5A) in response to current injection showed a similar trend in the I/O curve (Fig. 5B,C), with ACN-KO neurons unable to increase output spike frequencies following current injections to the levels shown by ACN-Ctr pyramidal cell. In comparison to their Ctr counterparts, ACN-KO CA1 neurons demonstrated lower intrinsic excitability and generated less AP on current injection. Interestingly, in this case no firing adaptation was observed in ACN-KO CA1 pyramidal cells, with no change in the AHP evident during the discharge. However, voltage-dependent  $Na^+$  and  $K^+$  currents (Fig. 5D) and the passive membrane properties (Table 2) did not differ between ACN-Ctr and ACN-KO. These observations strongly suggest that the impairment of neuronal excitability in ACN-KO CA1 neurons is also dependent on the dysregulation of extracellular  $K^+$  buffering, as described elsewhere (Meeks and Mennerick, 2004; Sibille et al., 2014; Chiacchiarretta et al., 2018).

Besides intrinsic excitability, basic excitatory synaptic transmission was also investigated by stimulating cerebellar mossy fibers or hippocampal Schaeffer collaterals and recording excitatory postsynaptic currents (EPSCs) in granule cells and CA1 neurons, respectively. However, no change in the basic excitatory synaptic

properties was detected in both brain regions (Suppl. Fig. 8), thereby confirming that astroglial CaN selectively modulates neuronal excitability.

### **3.4. CaN-dependent impairment of Na<sup>+</sup>/K<sup>+</sup> ATPase (NKA) activity in astrocytes of ACN-KO mice**

It is currently postulated that astrocytic NKA maintains extracellular K<sup>+</sup> homeostasis by clearing K<sup>+</sup> released during neuronal firing thereby preventing the fall of the gradient across neuronal membrane which sustains intrinsic excitability (Larsen et al., 2016). We, therefore, hypothesized that astrocytic CaN was able to modulate electro-responsiveness of CGCs and hippocampal CA1 neurons by regulating NKA activity and/or expression. In order to address these issues, we first measured ouabain-sensitive ATPase activity in CB and Hip lysates as well as in primary hippocampal astrocytes from ACN-KO mice. Specific NKA ATPase activity was significantly inhibited in both CB and Hip whole lysates by 62% and 78%, respectively (Fig. 6A,B). Furthermore, NKA ATPase activity was inhibited by 67% in purified astroglial (Fig. 6C), but not neuronal (Fig. 6D), cultures from the hippocampus of ACN-KO mice. Interestingly, in line with previous publications (Lea et al., 1994), NKA ATPase activity was inhibited by the CaN inhibitor FK506 in both total cerebellar lysates (Fig. 6A). However, when cell cultures were treated with FK506, NKA inhibition was observed only in astroglial cultures, but not in enriched neuronal cultures. (Fig. 6C and D) suggesting that the phosphatase activity of CaN is required for NKA activity in astrocytes but not in neurons.

To get further insight in mechanistic relationships between astroglial NKA and neuronal firing, we treated cerebellar and hippocampal slices with ouabain (100  $\mu$ M, 5 min) and registered neuronal discharges upon increasing depolarization steps. XXXXX (Fig. 6E,F).

Alongside direct dephosphorylation, CaN is known to regulate transcriptional activity of a number of genes in physiological as well as in pathological conditions. Transcriptional regulation may occur through direct activation of transcription factor of activated T-cells (NFAT) and by indirect activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) in astrocytes (Lim et al., 2013, 2016; Furman and Norris, 2014) and in other cell types (Hogan et al., 2003). NKA is composed of a larger catalytic alpha subunit and two smaller regulatory subunits (beta and gamma subunit) (Clausen et al., 2017). The functional core of NKA is composed of one alpha and one beta subunit, while the gamma subunit is required for fine tuning of NKA activity in tissue and isoform-specific manner (Geering, 2008). The alpha subunit includes four isoforms (coded by genes *Atp1a1-4*), of which three are expressed in brain (*Atp1a1-3*), the beta subunit includes three isoforms (coded by genes *Atp1b1-3*), of which two are expressed in brain. The heterodimer composed of alpha2 and beta2 is thought to be the astrocyte-specific variant, while the presence of alpha1 has also been shown (Larsen et al., 2016). We performed *in silico* analysis and found that the *Atp1a2* displays four high affinity NFAT binding sites, two of which are located in the proximal promoter region (-203 bp and -264 bp) and two locating in the distal promoter region (-1843 bp and -1998 bp) upstream of transcriptional start site

(Suppl. Fig. 9). Thus, a possible transcriptional effect of CaN on NKA expression and activity cannot be *a priori* ruled-out. Therefore, we employed real-time PCR to measure relative abundance of transcripts for Atp1a1 and Atp1a2 and Atp1b2 isoforms. We found, however, that during the entire period of brain development, specifically, at P0, P5, P10, P15, P20 and P30, in spite of strong developmental up-regulation (Orlowski and Lingrel, 1988) the levels of mRNA were not different between ACN-Ctr and ACN-KO (Fig. 7A). In line with this, protein levels of alpha1 and alpha2 NKA isoforms, as detected by WB, did not differ significantly neither in CB nor in Hip (Fig. 7B-E). Notably, despite their limited statistical sensitivity, these comparisons allow anyway to rule out biologically relevant effects (fold changes greater than 2, see figure caption). Last, no differences were found in the expression and localization of Atp1a2 NKA subunit in ACN-Ctr and ACN cerebella by immunohistochemical analysis (Suppl. Fig. 11). of These latter results suggest that activity modulation of NKA and not transcriptional regulation by CaN is important to control excitability of CB and Hip neurons by astrocytes. Inwardly rectifying K<sup>+</sup> channel 4.1 (Kir4.1), specifically expressed in astrocytes, may be involved in K<sup>+</sup> clearance by the mechanism of spatial buffering and maintenance of K<sup>+</sup> permeability in resting conditions. However, no changes were found in both protein and mRNA Kir4.1 expression levels between ACN-Ctr and ACN-KO astrocytes (Suppl. Fig. 10A,B).

Last, using whole-cell current-clamp recordings, we have also investigated whether pharmacological inhibition of CaN may affect resting membrane potential (RMP) in cultured astrocytes. The recording revealed a range of RMPs from  $-89.7$  to  $-23.8$  mV ( $n = 16$ ), with a median value of  $-70.7$  mV and an interquartile range IQR =  $[-76.1; -57.1]$  mV. This wide range of RMPs is in agreement with previous results obtained in rat hippocampal slice astrocytes (McKhann et al., 1997). Then we examined the effect of the inhibition of calcineurin activity on RMP. Overnight incubation of astrocytes with FK506 (200 nM) did not affect the median RMP, which resulted equal to  $-74.2$  mV with an IQR =  $[-78.1; -66.5]$  mV ( $n = 16$ ;  $p$ -value = 0.49, Mann-Whitney  $U$  Test, two-tail). Moreover, the marked heterogeneity of RMPs persisted in this condition, probably as an effect of a RMP bimodal distribution (McKhann et al., 1997; Bolton et al., 2006). In light of this, we focused our attention to the cellular subpopulation with a RMP more negative than  $-55$  mV, which also represents the majority of the cell sample. Again, we found no significant difference between control and treated astrocytes (mean  $\pm$  SEM in control astrocytes was  $-75.2 \pm 2.4$  mV ( $n = 12$ ); in treated astrocytes was  $-76.3 \pm 1.3$  mV ( $n = 12$ ;  $p$ -value = 0.69,  $t$ -test, two-tail).

### **3.5. Sodium homeostasis, glutamate uptake and gliotransmitter release in ACN-KO cultured astrocytes**

Astrocytes perform many housekeeping functions in the brain, many of which are regulated by sodium fluxed. In a first instance, these processes depend on the activity of NKA (Kirischuk et al., 2012; Verkhratsky et al., 2013). Therefore, it is logically to suggest that the described above impairment of neuronal excitability may be not only the effect of NKA inhibition produced by CaNB1 KO. We, therefore, made use of primary hippocampal cultures as well as primary purified astrocytes from ACN-Ctr and ACN-KO mice to investigate the link between astroglial CaN, the NKA activity and sodium-dependent astroglial functions. First, using immunocytochemistry and real-time PCR we investigated expression of general

astroglial markers like GFAP and glutamine synthetase (GS). Supplementary Figure XXX shows that both ACN-Ctr and ACN-KO astrocytes express GFAP and GS with no differences in protein expression, localization or mRNA levels of both markers. Next, we have investigated sodium homeostasis and the expression and activity of the sodium-dependent transporter in primary cultured astrocytes from ACN-Ctr and ACN-KO mice. First, using a sodium-sensitive fluorescent dye SBFI, we measured intracellular sodium concentration and found it significantly higher in ACN-KO ( $31.62 \pm 2.36$  mM,  $n = 279$ ) than in ACN-Ctr astrocytes ( $9.1 \pm 1.01$  mM,  $n = 343$ ,  $p < 0.0001$ ) (Fig. 8A) suggesting that the sodium-dependent transport may be altered. However, the glutamate uptake rate was not different between ACN-KO ( $XXX \pm XXX$  mM) and ACN-Ctr cells ( $XXX \pm XXX$  mM,  $p = XXX$ ) (Fig. 8B). This may be explained by differences in the glutamate transporters expression. In our previous study (Ruffinatti et al., 2018) we found that, in cultured astrocytes, glutamate/aspartate transporter (GLAST) is predominantly expressed over glutamate transporter 1 (GLT-1) (Fig. 8C), so we investigated GLAST expression using WB analysis. Fig. 8D shows that GLAST is expressed significantly higher in ACN-KO than in ACN-Ctr astrocytes, which may compensate the intracellular  $\text{Na}^+$  increase and the decrease in  $\text{Na}^+$  driving force for glutamate uptake. Next we measured release of metabolites, such as lactate and gliotransmitter glutamate. While there were no differences in the release of lactate ( $XXX \pm XXX$  mM in ACN-Ctr vs  $XXX \pm XXX$  mM in ACN-KO,  $p = XXX$ ), the release of glutamate was significantly lower in ACN-KO ( $XXX \pm XXX$  mM) than in ACN-Ctr astrocytes (Figures 8E and 8F, respectively).

Finally, we have investigated reactive status of astrocytes measuring the levels of microglial reactivity marker Iba1 and inflammation-related cytokines IL-1 $\beta$  and TNF $\alpha$ . No significant differences were found in mRNA levels of Iba1, IL-1 $\beta$  and TNF $\alpha$  (Supplementary Figure XXX).

Collectively, these data suggest that there may be alterations in sodium-regulated processes in ACN-KO astrocytes, although functional consequences of these alterations for the astroglial homeostatic functions remain to be investigated.



#### 4. DISCUSSION

In the present manuscript, we investigated for the first time the role of astrocytic CaN in the healthy brain. We report that the specific deletion of CaNB1 from GFAP-expressing astrocytes during the postnatal period: 1) does not alter development of ACN-KO mice and of the morphology of the central nervous system (CNS); 2) does not have impact on the number of hippocampal NPCs; 3) severely impairs intrinsic excitability in granule cerebellar neurons and hippocampal CA1 pyramidal neurons; and 4) that this the impairment is likely due to the lack of positive regulation of NKA by calcineurin in astroglia.

To generate the ACN-KO mouse, we used a 77.6 GFAP-Cre mouse line recently reported and deposited to Jackson Lab mouse repository by Michael Sofroniew (JaxLab Stock No. 024098). To date, several GFAP-Cre lines are commercially available, which differ in cellular specificity and developmental pattern of Cre expression (Zhuo et al., 2001; Garcia et al., 2004; Gregorian et al., 2009). Recently, Fujita and colleagues (Fujita et al., 2018) reported the generation of a CaN KO using Cre expression driven by the a 2.2 kb 5' flanking fragment of the human GFAP gene generated by (Zhuo et al., 2001). The authors observed alterations of early postnatal development and premature death of pups around 1 mo of age. The authors concluded that the deletion of CaNB1 from glial cells of the enteric nervous system resulted in strong inflammation and mucosal permeability in small intestine which led to an inability to digest and absorb food (Fujita et al., 2018). In preliminary experiments in our lab, we observe expression of tdTomato reporter also in enteric glial cells, thereby confirming this localization and the ablation of calcineurin in these cells also in our model. Yet, we do not confirm the effect on growth and development. It has to be noted that the GFAP-Cre line used by Fujita et al. displays embryonic (E13.5) onset of Cre expression and in adulthood is also expressed in oligodendroglia, ependymal cells, some neurons in the CNS as well as in periportal liver cells (Zhuo et al., 2001) introducing thus a bias in the interpretation of the results. For example, neuronal CaN plays a crucial role in axonal growth and guidance during vertebrate development by activating NFAT (Nguyen and Di Giovanni, 2008). Also the expression in pre-natal periods might explain the difference. For example, a recent study demonstrated that early embryonic exposure to cyclosporine, another established CaN inhibitor, causes a reduction in eye and brain size in zebrafish (Clift et al., 2015). Although it has been stated that 77.6 GFAP-Cre mouse line does not have Cre expression in neuronal progenitors in the hippocampus (Gregorian et al., 2009), we found partial co-localization of a marker of neuronal stem cells, Sox2, with tdTomato reporter at P7, a short temporal overlap between the end of the embryonic and perinatal Sox2 expression and the onset of GFAP-Cre expression in ACN-KO mice. Nevertheless, this did not have resulted Cre expression in adult neurons, as at 1 mo of age we have found only single cells in the DG with were positive for both, NeuN, a marker for differentiated neurons and tdTomato reporter. Moreover, there were no differences in the number of double positive Sox2/tdTomato or NeuN/tdTomato cells, and the number of neuronal progenitors in adulthood was not different.

A central mechanistic finding of this report is that selective deletion of astrocytic CaNB1 severely impaired intrinsic excitability in CGCs and hippocampal CA1 neurons. It has long been known that alterations of astrocyte capacity to clear extracellular K<sup>+</sup> result in dramatic changes in neuronal network excitability, which

may result in neurological disorders, including epilepsy, Rett syndrome, Alzheimer's Disease and Huntington's Disease (Meeks and Mennerick, 2004; Sibille et al., 2014; Chiacchiaretta et al., 2018). Originally, two mechanisms were proposed for astrocytes to buffer extracellular  $K^+$ : (i) the net  $K^+$  uptake, an energy-dependent  $K^+$  buffering mediated by glial NKA (Hertz, 1965, 1979) and (ii) the  $K^+$  spatial buffering, an energy-independent re-distribution of  $K^+$  from regions with high  $K^+$  to those with low  $K^+$  concentrations (Orkand et al., 1966). Subsequent work showed that the  $K^+$  spatial buffering involved  $K_{ir}$  channels for local  $K^+$  accumulation and gap junctions for redistribution of  $K^+$  (Hertz and Chen, 2016). Currently, it is postulated that astrocytic  $K_{ir}$ -mediated spatial buffering is mainly responsible for resting  $K^+$  permeability (Neusch et al., 2001; Djukic et al., 2007; Kucheryavykh et al., 2007), while  $K^+$  clearance during neuronal firing is mainly due to NKA activity. Notably, analysis of  $K^+$  affinity of heterodimers with different alpha and beta subunits composition revealed that the astroglial alpha2-beta2 combination has the lowest  $K^+$  affinity (Larsen et al., 2014) and therefore is particularly suitable for the removal of large amounts of  $K^+$  released from neurons during intense neuronal activity (Larsen et al., 2016). The following evidences strongly support the hypothesis that the inhibition of astrocytic NKA impairs neuronal excitability in ACN-KO mice. First, NKA ATPase activity was strongly inhibited in hippocampal and cerebellar tissues and in purified astroglial cultures but not in enriched neuronal cultures from ACN-KO pups. This finding demonstrates that astrocytic NKA is selectively inhibited in the brain of ACN-KO mice. Second, NKA activity was significantly impaired by FK506 in both total cerebellar lysates and in cultured astrocytes from WT animals. This finding demonstrates that CaN activation is permissive towards astrocytic NKA activity. This latter observation supports reports by others in cells other than astrocytes (Marcaida et al., 1996; Mallick et al., 2000; Woolcock and Specht, 2006; Rambo et al., 2012) as well as in kidney tissue preparations (Tumlin and Sands, 1993; Lea et al., 1994). It is postulated that CaN-dependent dephosphorylation activates, while phosphorylation, *e.g.* by protein kinase A (PKA) or protein kinase C (PKC), inhibits NKA activity (Bertorello et al., 1991; Bertuccio et al., 2003, 2007; Poulsen et al., 2010). Third, the lack of significant changes in their resting membrane potential indicates that deletion of astrocytic CaN did not alter the transmembrane  $K^+$  gradient in cerebellar and hippocampal neurons, which further confirms that neuronal NKA activity was unaltered. Last, treatment of slices with ouabain ...

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It should be acknowledged that only the astrocyte-specific knock-down or knock-out of NKA would unequivocally rule-out the involvement of neuronal NKA and other  $K^+$  transporters, *e.g.*  $K_{ir}$  channels, in the observed inhibition of neuronal excitability in ACN-KO mice. However, such experiments might be challenging because, at worse, complete elimination of NKA ATPase activity from astrocytes would require knocking down of at least two NKA isoforms, namely alpha1 and alpha2. This rationale is based on an assumption that both alpha1 and alpha2 NKA are inhibited by deletion of CaNB1 in ACN-KO astrocytes. Even if the lower  $K^+$  affinity of alpha2-beta2-composed NKA (Larsen et al., 2014, 2016) suggests the predominant involvement of alpha2 isoform in  $K^+$  clearance, the contribution of alpha1 subunit cannot presently be ruled-out. Moreover, cultured purified astrocytes tend to lose the specific alpha2 isoform, thereby making the alpha1

the predominant isoform (Peng et al., 1998). For these considerations, we did not investigate which NKA isoform is activated by astrocytic CaN, acknowledging this as a limitation of the present work.

In addition to regulation of target proteins by dephosphorylation, CaN regulates protein expression by modulating gene transcription, e.g. through direct interaction with transcription factor NFAT in many cell types (Hogan et al., 2003), including astrocytes (Furman and Norris, 2014). The presence of several high score NFAT-binding sites in the promoter of *Atp1a2* gene, detected by our *in silico* analysis, suggests that both of these mechanisms may potentially concur for the control of NKA activity in astrocytes. The hypothesis of transcriptional regulation of NKA by CaN is also supported by extensive data on the yeast analog of NKA, ENA1, in which stress-induced expression of ENA1 is mediated by CaN through activation of the transcription factor Crz1 (Mendoza et al., 1994; Mendizabal et al., 2001; Ruiz et al., 2003; Ruiz and Ariño, 2007). Nevertheless, our data demonstrate that, in the current experimental setting, mRNA levels of both *Atp1a1* and *Atp1a2* were not different throughout the postnatal brain development period, a finding which rules-out transcriptional regulation. In agreement with this assertion, protein levels of both  $\alpha 1$  and  $\alpha 2$  NKA were not different in hippocampal and cerebellar tissues of ACN-Ctr and ACN-KO mice.

The regulation of neuronal excitability by astrocytic CaN reinforces the emerging evidence in favor of bidirectional communication between neurons and astrocytes (Durkee and Araque, 2019). Recently, we have proposed that neuronal activity may lead to CaN activation in non-neuronal cells (Lim et al., 2016) as it was shown for other cells, e.g. in pericytes (Filosa et al., 2007) and in brain microvascular endothelial cells (Zuccolo et al., 2017). Furthermore, we have previously demonstrated that chemical induction of neuronal activity robustly activates CaN in astrocytes juxtaposed to activated neurons in *in vitro* co-cultures (Lim et al., 2018). Based on pharmacological characterizations, we have suggested that these effects are mediated by glutamate through astroglial mGluR5 and store-operated  $\text{Ca}^{2+}$  entry mechanism (Lim et al., 2018).

In summary, we present here a conditional KO of CaN in astrocytes suitable for investigating the role of CaN in the CNS physiology. Initial characterization of the mouse model led to establish that this enzyme in astrocytes regulates NKA activity and that neuronal excitability is altered both in the cerebellum and in the hippocampus. From these data, a hypothetical model can be generated in which neurons signal to astrocytes and this leads to astrocytic  $\text{Ca}^{2+}$ -rises that are sufficient to activate calcineurin, which in turn controls  $\text{K}^+$  transport and feeds back to neuronal excitability. More generally, our data show that astrocytes are key regulators of neuronal activity.

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## LEGENDS

**Figure 1. PCR characterization of ACN-KO mice.** **A**, RT-PCR on whole cerebellar tissue using primers spanning exons 2-6 of CaNB1 mRNA. Alongside the 517 bp amplicon present in both WT and KO, the appearance of 95 bp amplicon in ACN-KO tissue indicates deletion of exons 3-5. Primers amplifying the deleted exons 3-5 (148 bp) show the presence of amplicons in both ACN-Ctr and ACN-KO tissues (**B**), while Cre amplicon (143 bp) was present only in ACN-KO cerebellum (**C**). Representative PCRs are shown from at least 4 independent litters. S18 mRNA (108 bp) was used as loading control. **D**, Real-time PCR on FACS sorted Draq5<sup>+</sup>/GLAST<sup>+</sup>/CD90.2<sup>-</sup> astrocytes from cerebellum of P20 ACN-KO mice (mean  $\pm$  SD, n = 3). Primers were used to amplify specifically exons 3-5 which are deleted in ACN-KO astrocytes (light-grey arrow). **C** and **D**, Anti-CaNB1 western blot analysis of purified cultures of cerebellar (**A**) and hippocampal (**B**) astrocytes shows strong reduction of CaNB1 protein in ACN-KO astrocytes while mixed neuron-astroglial primary cultures still retain calcineurin presence (**C**). Data are presented as mean  $\pm$  SEM of 3-4 independent tissue preparations.

**Figure 2. Normal growth and brain weight of ACN-KO mice, and time course of CaNB1 KO development in the cerebellum.** Body weight until 8 mo of age (**A**) and brain weight at one month of age (**B**) were not different between ACN-Ctr and ACN-KO mice. In **A**, data are expressed as mean  $\pm$  SEM. The number of mice per age was 6-20. In **B**, data are expressed as mean  $\pm$  SEM of 11 (ACN-Ctr) and 10 (ACN-KO) brains. **C**, Time course of tdTomato expression in the cerebellum of RATO-ACN-KO mice shows appearance of reporter protein between 7 and 10 postnatal days. Full and widespread CaNB1 KO in the cerebellum is achieved by P15-P20. Images are representative from at least 3 ACN-KO mice for each age-point.

**Figure 3. Adult neurogenesis and synaptic proteins are not altered in ACN-KO mice.** **A**, Number of DCX<sup>+</sup> cells was similar in ACN-Ctr compared to ACN-KO mice (n=4 per group) in both subgranular zone (SGZ; MDE:  $d=2.38$ ,  $\Delta=47$  cells) and granular cell layer (GCL; MDE:  $d=2.38$ ,  $\Delta=172$  cells). **B**, Similarly, the number of BrdU<sup>+</sup>/NeuN<sup>+</sup> cells was not significantly different between ACN-Ctr and -KO mice (n=5 per group) in neither of these two brain regions (MDE in SGZ:  $d=2.02$ ,  $\Delta=53$  cells; MDE in GCL:  $d=2.19$ ,  $\Delta=252$  cells). Western blot analysis using anti PSD95 or anti-synaptophysin (Sy38) antibody revealed no differences in whole cerebella and hippocampal tissues (**C**), and in hippocampal mixed neuron-astroglial primary cultures (**D**). In **C** and **D**, data are expressed as mean  $\pm$  SD of 3-4 independent brain or culture preparations.

**Figure 4. Evaluation of the intrinsic excitability of cerebellar granule cells in ACN-Ctr and ACN-KO mice.** **A**, Schematic representation of a cerebellar circuit which illustrates how mossy fibers (MF) contact cerebellar granule cells (CGCs) and Golgi cell (GoC) dendrites. The basic electroresponsiveness of CGCs was investigated through the whole-cell patch-clamp technique. **B**, Granule cell electroresponsiveness. Voltage responses were elicited from -70 mV using step current injection. **C**, The plot shows the relationship between average firing frequency over 2 sec and the injected current intensity (F-I) both for ACN-Ctr (n = 5) and ACN-KO (n = 5) granule cells. Data are reported as mean  $\pm$  SEM. **D**, The bar chart shows the % change of AHP between the first and the last spike for both ACN-Ctr (n = 31) and ACN- KO (n = 18). **E**, The bar chart compares voltage-gated inward (i.e. Na<sup>+</sup>) and outward (i.e. K<sup>+</sup>) current amplitudes measured at -40 mV and

+20 mV in ACN-Ctr (n = 6) and ACN-KO (n = 6) mice. No significant difference was detected in none of the performed comparisons. MDE for inward Na<sup>+</sup> currents:  $d=1.80$ ,  $\Delta=1195.7$  pA. MDE for transient outward K<sup>+</sup> currents:  $d=1.80$ ,  $\Delta= 828.4$  pA. MDE for persistent outward K<sup>+</sup> currents:  $d=1.80$ ,  $\Delta= 731.5$  pA.

**Figure 5. Evaluation of the intrinsic excitability of hippocampal CA1 neurons in ACN-Ctr and ACN-KO mice.** **A**, Schematic representation of the hippocampus circuit. The basic electroresponsiveness of CA1 pyramidal neurons was investigated through the whole-cell patch-clamp technique. **B**, Electroresponsiveness of hippocampal CA1 pyramidal neurons. Voltage responses were elicited from -80 mV using step current injection. **C**, Relations between injected current and average firing frequency (F–I) for ACN-Ctr (n=9) and ACN-KO (n=11) CA1 pyramidal cells. Differences between the two F-I curves were statistically significant within the 325-400 pA range,  $p = 0.001$ . Data are reported as mean  $\pm$  SEM. **D**, The bar charts compare inward (i.e. Na<sup>+</sup>) and outward (i.e. K<sup>+</sup>) current amplitudes measured at -40 mV and +20 mV in ACN-Ctr and ACN-KO mice. Data are reported as mean  $\pm$  SEM.

**Figure 6. NKA ATPase activity is decreased in tissues and in cultured astrocytes of ACN-KO mice.** Ouabain-sensitive ATPase activity was measured in whole tissue cerebellar (**A**) and hippocampal (**B**) lysates as well as in purified astroglial primary cultures (**C**) and in enriched neuronal cultures (**D**) from ACN-Ctr and ACN-KO mice. Where indicated FK506 was added to the lysates at a final concentration of 200 nM. NKA ATPase activity is expressed as pmol of inorganic phosphate per  $\mu$ g total proteins measured in 1 minute (pmol/ $\mu$ g/min). Data are expressed as mean  $\pm$  SEM from 3-5 independent tissue or culture preparations.

## OUABAIN

**Figure 7. Transcripts and protein levels of astrocytic NKA isoforms are not different between ACN-Ctr and ACN-KO mice.** mRNA levels of catalytic Atp1a1, Atp1a2 and regulatory Atp1b2 isoforms were measured by real-time PCR in hippocampal whole tissue lysates at indicated time points during the first month of post-natal development (**A**). Data are expressed as mean  $\pm$  SEM of 3-6 hippocampal lysates. At each time-point, the differences were not significant between ACN-Ctr and ACN-KO. **B**, Western blot analysis could not detected any significant differential expression in whole tissue lysates from ACN-Ctr mouse cerebellum compared to ACN-KO neither for Atp1a1 (MDE:  $d=1.40$ ,  $\Delta=1.20$ ) nor for Atp1a2 catalytic isoforms (MDE:  $d=1.41$ ,  $\Delta=0.88$ ). **C**, Similarly, the differential expression of these two catalytic isoforms was not even significant when evaluated in hippocampus (MDE for Atp1a1:  $d=1.71$ ,  $\Delta=1.05$ ; MDE for Atp1a2:  $d=1.71$ ,  $\Delta=0.73$ ). Representative bands are shown of 6-10 independent experiments.

**Figure 8. Intracellular sodium and sodium-dependent transport in ACN-KO astrocytes.** **A**, intracellular [Na<sup>+</sup>] was measured using SBFI probe in cultured hippocampal astrocytes. Data are expressed as mean  $\pm$  SEM of n = 343 cell (ACN-Ctr) and n = 279 cells (ACN-KO) from 5 independent cultures for each genotype performed in triplicate. **B**, glutamate uptake. **C**, Western blot analysis of GLAST expression. **D** and **E**, glutamate and lactate release, respectively.

mRNA levels of catalytic Atp1a1, Atp1a2 and regulatory Atp1b2 isoforms were measured by real-time PCR in hippocampal whole tissue lysates at indicated time points during the first month of post-natal development

(A). Data are expressed as mean  $\pm$  SEM of 3-6 hippocampal lysates. At each time-point the differences were not significant between ACN-Ctr and ACN-KO. Protein levels of Atp1a1 and Atp1a2 catalytic isoforms were analyzed by Western blot in whole tissue lysates from cerebellum (B) and hippocampus (C). Representative bands are shown of 6-9 independent experiments. Densitometric data are expressed as mean  $\pm$  SD.

**Table 1. Passive properties of cerebellar granule cells.**

	ACN-Ctr (n=6)	ACN-KO (n=6)	p-value
<b>C<sub>m</sub> (pF)</b>	2.67 ± 0.19	2.53 ± 0.13	
<b>R<sub>in</sub> (GΩ)</b>	2.90 ± 0.30	3.06 ± 0.60	
<b>V<sub>rest</sub> (mV)</b>	-55.4 ± 3.34	-47.2 ± 2.97	

**Table 2. Passive properties of hippocampal CA1 pyramidal cells.**

	ACN-Ctr (n=4)	ACN-KO (n=4)	p-value
<b>C<sub>m</sub> (pF)</b>	126.81±8.08	102.18 ±5.00	
<b>R<sub>in</sub> (GΩ)</b>	0.222±0.03	0.256±0.008	
<b>V<sub>rest</sub> (mV)</b>	-52.2±4.24	-55.7 ± 2.26	